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Review

Molecular aspects of Cu, Fe and Zn homeostasis in plants

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Abstract

Proper metal transport and homeostasis are critical for the growth and development of plants. In order to potentially fortify plants pre-harvest with essential metals in aid of human nutrition, we must understand not only how metals enter the plant but also how metals are then delivered to the edible portions of the plant such as the seed. In this review, we focus on three metals required by both plants and humans: Cu, Fe and Zn. In particular, we present the current understanding of the molecular mechanisms of Cu, Fe and Zn transport, including aspects of uptake, distribution, chelation and/or sequestration.

Keywords: Iron; Zinc; Copper; Transport; Regulation; Chelation

1. Introduction

Humans require 17 essential minerals, all of which can be obtained through a proper diet. Plants also require most of these same essential minerals, all of which are obtained from the soil. Deficiencies of essential minerals affect both the quality and quantity of plant foodstuffs, thereby negatively affecting a large portion of the world's population for whom plants serve as the major dietary source of essential minerals. Engineering plants with enhanced mineral content could potentially alleviate current problems of human mineral deficiency by enhancing crop yields and/or fortifying plants pre-harvest. However, before we can increase the mineral content of plants, we need to understand not only how minerals are obtained from the soil but how the minerals are then distributed throughout the plant. In particular, in order to address problems of human mineral deficiencies through plants, we need to ensure that the desired minerals are accumulating in the edible portions of the plant.

In this review, we focus on current knowledge of how plants obtain and distribute three important divalent cations: Cu, Fe and Zn. These metals share some common features with each other. They are all necessary nutrients yet they are toxic at high concentrations. Thus, plants must balance uptake, utilization and storage of these metals in order to maintain proper ion homeostasis. Although each of these metals can be transported by a variety of transporters, some transport all three and some show specificity for a particular ion. Different strategies for chelation and storage are also emerging.

2. Iron

The WHO estimates two billion people suffer from some form of Fe deficiency anemia, making Fe deficiency the leading human nutritional disorder in the world today [1]. In addition, although Fe is the fourth most abundant element in the earth's crust, it is the third-most limiting nutrient for plant growth primarily due to the low solubility of Fe in aerobic environments [2]. In fact, over one third of the world's soils are considered Fe deficient. In order to deal with the limiting amounts of Fe, plants have evolved several strategies to obtain Fe from the soil [3–5]. The Strategy I mechanism includes proton extrusion to solubilize Fe(III) in the soil, reduction of the solubilized Fe(III) by a membrane-bound Fe(III) chelate reductase and subsequent transport of the resulting Fe(II) into the plant root cell by a Fe(II) transporter [6]. Strategy II is a chelation-based strategy involving release of Fe(III)-specific phytosiderophores (PS) and subsequent uptake of the Fe(III) phytosiderophore complexes via a specific transport system. Each will be briefly described below.

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2.1. Iron uptake from the soil using Strategy I

2.1.1. Proton release

For every one unit drop in pH, Fe becomes a thousand fold more soluble. Thus, in order to lower the rhizosphere pH and increase the amount of soluble Fe(III), Strategy I plants extrude protons in response to Fe deficiency [7]. Although the protein responsible for releasing protons into the rhizosphere has not yet been identified, several proton-ATPases of the AHA (*A*rabidopsis H^+-*A*TPase) family are induced in the roots of Fe-deficient plants ([8] and Guerinot lab, unpublished data). Likewise, in tomato roots, increased proton extrusion is detected in response to Fe deficiency, and a monoclonal antibody directed against a P-type proton-ATPase from maize reacts with more proteins from Fe-deficient roots than from Fe-sufficient roots [9].

2.1.2. Reduction of Fe(III)

FRO2 is the enzyme responsible for the plasma membrane Fe(III) chelate reductase activity that is induced under iron deficiency in the roots of Arabidopsis [10]. Furthermore, overexpression of *FRO2* has demonstrated that Fe(III) chelate reductase activity is the rate-limiting step in Fe acquisition; plants overexpressing *FRO2* are resistant to low Fe growth conditions [11]. In Arabidopsis, FRO2 is regulated both transcriptionally and post-transcriptionally. In *FRO2* over-expressing plants, although *FRO2* mRNA is detected under both Fe-sufficient and Fe-deficient conditions, Fe(III) chelate reductase activity is present only in Fe-deficient roots [11]. In Arabidopsis, *FRO2* belongs to an eight-member gene family [12,13]. Although *FRO2* is expressed exclusively in the roots, some of the *FRO* genes are expressed specifically in the shoots (*FRO6*, *FRO7* and *FRO8*). Additionally, *FRO6* is regulated by light; its promoter contains multiple LREs (*L*ight *R*esponsive *E*lements) and strong GUS staining is detected in light-grown seedlings carrying a *FRO6* promoter-GUS transgene while etiolated seedlings carrying the same transgene show no GUS staining [13].

The Fe(III) chelate reductase responsible for root Fe acquisition in pea has also been identified [14]. *PsFRO1* mRNA, like *AtFRO2* mRNA, accumulates in Fe-deficient roots. However, although *PsFRO1* mRNA appears to be at the highest levels in the epidermal cells using in situ hybridization, it is seen throughout the root whereas *AtFRO2* is only detected in the epidermal cells. Consistent with the regulation of *FRO2* mRNA accumulation in Arabidopsis, *LeFRO1* mRNA accumulates in Fe-deficient roots of tomato [15]. However, unlike *FRO2*, *LeFRO1* mRNA is also detected in shoots regardless of Fe status, suggesting that *LeFRO1* may play a role in Fe mobilization in the shoots as well. *LeFRO1* localizes to the plasma membrane in onion epidermal cells and confers Fe(III) reductase activity when expressed in yeast.

2.1.3. Transport of Fe(II)

Once Fe is reduced, it is transported across the plasma membrane of root epidermal cells by IRT1 (*I*ron *R*egulated *T*ransporter) [16]. IRT1 is a member of the ZIP (*Z*RT *I*RT-like *P*rotein) family [17], and it is the major root Fe transporter in Arabidopsis [18–20]. Orthologs of IRT1 have also been characterized in tomato and rice; the mRNAs of both genes, like *IRT1*, accumulate in Fe-deficient roots [21–23]. Although IRT1 is able to mediate the transport of multiple metals in yeast, including Fe, Mn, Zn and Cd, [16,24], Fe appears to be the most important metal for IRT1 *in planta*. *irt1* plants have reduced levels of Fe, Mn, Zn and Co, but they die before setting seed unless supplied with Fe [18]. *IRT2* is also expressed in the epidermal cells of Fe-deficient roots, and it is able to transport some of the same metals as IRT1, namely Fe and Zn [25]. However, the overexpression of *IRT2* in *irt1* mutants is not able to rescue the phenotypes observed in *irt1* mutants, indicating that the two proteins do not have redundant functions [18,19].

2.2. Iron Uptake from the soil using Strategy II

The grasses are Strategy II plants, and they use a chelation-based strategy to acquire Fe [26]. In response to Fe deficiency, Fe(III)-binding compounds termed phytosiderophores are released. One class of these low molecular mass compounds are known as mugineic acids (MA); they bind soluble Fe and then are taken back up into the roots of the plant via an Fe–PS transporter. Most of the genes involved in the biosynthesis of phytosiderophores have been cloned and will not be reviewed here (for a review on this topic see [3]).

Until recently, Strategy II plants were thought to only use the above-described response to obtain Fe from the soil [23]. Rice, however, in addition to having the ability to transport Fe(III)–PS complexes, is also able to transport Fe(II), which is perhaps not surprising because rice has orthologs of IRT1 as well putative Fe (III) chelate reductases. It makes sense that rice would be able to induce both responses since rice grows in water-logged soils that tend to be rich in Fe(II); currently it is not known whether other Strategy II plants which grow in soils more limited for Fe(II) are also capable of inducing the Strategy I response.

The Fe–PS transporter involved in the Strategy II response in maize, YS1 (*Y*ellow *S*tripe), was identified through the cloning of the gene defective in the *ys-1* mutant, which has interveinal chlorosis and is unable to mediate Fe–PS uptake [27,28]. YS1 is an integral membrane protein that can transport Fe–PS in yeast, and *YS1* mRNA accumulates in response to Fe deficiency [27]. Furthermore, *YS1* is expressed in both the roots and the shoots suggesting a role for YS1 not only in the primary uptake of Fe–PSs but in the intercellular transport of Fe in the shoots of the plant as well.

2.3. The intercellular and intracellular transport of Fe

After Fe has been transported across the plasma membrane of the epidermal cells in roots, members of several different transport families have been implicated in the intracellular and intercellular transport of iron, including the Nramp (*N*atural *r*esistance *a*ssociated *m*acrophage *p*rotein) family and the YSL (*Y*ellow *S*tripe-*L*ike) family. The Nramp family transports divalent cations and the YSL family members likely transport metal chelates. Each will be briefly described here.

2.3.1. Nramp

The Nramp family is an evolutionarily conserved, ubiquitous metal transport family. Characterized mammalian Nramps mediate the transport of metal into the cytoplasm, and DMT1 (*D*ivalent *M*etal *T*ransporter) is responsible for dietary Fe absorption [29]. In Arabidopsis, there are seven members of the NRAMP family, three of which have been implicated in Fe transport [17,30]. AtNRAMP1, AtNRAMP3 and AtNRAMP4 all mediate the transport of Fe in yeast based on their abilities to complement an Fe uptake mutant [31,32]. These genes can also mediate the transport of Mn and Cd in yeast because they can functionally complement a Mn-uptake defective mutant as well as confer Cd sensitivity to wild-type yeast [32]. *In planta*, mRNAs of *AtNRAMP1*, *AtNRAMP3* and *AtNRAMP4* accumulate in response to Fe deficiency; *AtNRAMP1* and *AtNRAMP3* accumulate mainly in the roots and *AtNRAMP4* accumulates in both the roots and shoots. A function in metal transport has also been demonstrated *in planta*. The overexpression of *AtNRAMP1* increases the resistance of plants to growth at toxic Fe concentrations suggesting a role in Fe distribution rather than uptake [31]. AtNRAMP1 has a plastid targeting sequence, and as plastids are a site of Fe storage in plants [33], it is possible that AtNRAMP1 functions to transport excess Fe into the plastids as a means of preventing toxicity. This would require that AtNRAMP1 mediate metal transport out of the cytoplasm, however, a direction different than AtNRAMP3 and AtNRAMP4.

AtNRAMP3 and AtNRAMP4 have been demonstrated to play a role in the intracellular transport of Fe, specifically in mobilizing Fe from the vacuole. AtNRAMP3 localizes to the vacuolar membrane and *AtNRAMP3* overexpression leads to the down-regulation of *IRT1* and *FRO2* mRNA, both of which accumulate in response to Fe deficiency [34]. These data suggest that the overexpression of *AtNRAMP3* leads to increased Fe levels in the cytosol thereby downregulating Fe deficiency-induced genes such as *FRO2* and *IRT1*.

NRAMP4, like *NRAMP3*, localizes to the vascular system of the roots and shoots of Arabidopsis [34,35]. At the subcellular level, NRAMP4, like NRAMP3, localizes to the vacuolar membrane [35,36]. The similar expression patterns at both the subcellular and tissue level suggest that NRAMP3 and NRAMP4 function redundantly [35]. In fact, although the single mutants of each gene do not have obvious growth defects, the double mutant is sensitive to Fe depletion with the severity of the phenotype being correlated with the level of Fe depletion. This is best demonstrated using calcareous soils which are low in Fe. Although the double mutant grows well in humic (Fe-sufficient) soils, there is a greater than 90% lethality rate for *nramp3 nramp4* double mutant plants grown in calcareous soils. This phenotype is believed to be due to an inability of the double mutants to mobilize Fe stores from the vacuole early in development. Using electron microscopy, the authors demonstrated Fe localization to globoids in the seeds of both wild-type and mutant plants. By day 2, although almost no Fe was detected in the globoids of wild-type plants, Fe was still clearly visible in the globoids of mutant plants, suggesting a defect in the ability to mobilize Fe from the globoids. What still remains to be determined is the relative contribution of Fe storage in the vacuole versus Fe storage in ferritin which is predominantly found in plastids [37]. Ferritin is encoded by a four-member gene family in Arabidopsis, members of which show differential patterns of expression [38].

It is clear that if Fe needs to be mobilized from the vacuole that there must be a transporter responsible for moving Fe into the vacuole. In yeast, *CCC1* (*C*a^{2+}-sensitive *c*ross-*c*omplementer) encodes a transporter that can mediate the transport of Fe and Mn into vacuoles [39]. Yeast overexpressing *CCC1* have an increased concentration of vacuolar Fe, and conversely, deletion mutants accumulate less Fe in their vacuoles and are sensitive to Fe toxicity. An ortholog of CCC1 has been identified in Arabidopsis (Guerinot lab, in preparation). The protein encoded by this gene, VIT1 (*V*acuolar *I*ron *T*ransporter), is 58% similar to CCC1, and the expression of *VIT1* in a Δ*ccc1* mutant rescues the ability of the mutant to grow in the presence of high Fe. *In planta*, VIT1 is hypothesized to oppose the function of NRAMP3 and NRAMP4 in that it would transport Fe into the vacuole while NRAMP3 and NRAMP4 are thought to mobilize Fe out of the vacuole. Consistent with VIT1 playing an opposing role, *VIT1* is also expressed in the vasculature of the roots and shoots.

2.3.2. YSL family

The YSL family, consisting of eight members in Arabidopsis, has been implicated in the intercellular transport of Fe chelates, specifically Fe complexed to nicotianamine (NA) [27]. We will first discuss the transporter family and then discuss the chelator itself.

Several lines of evidence have led to the prediction that one of the YSL family members, YSL2, functions in the lateral movement of Fe(II)–NA complexes within the veins. First, in yeast, YSL2 is able to mediate the transport of Fe(II)–NA as well as Cu–NA [40]. Second, a *YSL2* promoter GUS fusion results in vascular staining in the roots and the shoots. Lastly, when GFP (Green Fluorescent Protein) is fused to the C-terminus of YSL2, fluorescence is detected along the lateral membranes of the root consistent with lateral transport. In contrast, very few cells show any apical or basolateral membrane staining. The function of YSL2 is only proposed, however; a T-DNA insertion has been identified in *YSL2*, but *ysl2* mutant plants do not display any obvious phenotypes. Thus it is likely that YSL2 is functionally redundant with another protein. Furthermore, the data of a second group contradict some of the above-described data [41]. More specifically, this group has been unable to demonstrate Fe(II)–NA transport by YSL2 in yeast. In fact, they suggest that the results of the other group are perhaps due to differences in the vectors used. However, the two groups did not use the same growth conditions so it is hard to compare their results. In addition, the second group detects GUS staining in the roots of all of their lines but only observes shoot staining in a few of their lines. This observation is consistent with their Northern analysis in which *YSL2* is only detected in the roots. Thus, given the discrepancies, the exact role of YSL2 in Fe distribution remains unclear.

A loss-of-function mutant has been identified in *YSL1* [42], and the phenotype of this mutant supports a role for YSL1 in the transport of Fe–NA complexes, especially in the delivery of Fe and NA to the seed. Although there are no obvious growth defects or changes in metal content, these mutants accumulate 40% more NA in the shoots. Conversely, the seeds of *ysl1* mutants contain lower amounts of Fe–NA than wild type, a phenotype that cannot be rescued by the addition of exogenous Fe. Consistent with a change in Fe–NA levels in *ysl1* seeds, there is a transient defect in the germination of these seeds that can be rescued by the addition of Fe to the media. Presumably YSL1 functions not only in the delivery of Fe–NA to the seeds but also in the long distance transport of Fe–NA as evidenced by the *YSL1* expression pattern. When the *YSL1* promoter drives the expression of *uidA*, GUS staining is detected in the veins of the shoots, the vasculature of the sepals and petals as well as in the siliques and pollen grains.

The *YSL* genes also play a role in Strategy II plants. In fact, 18 *YSL* genes have been identified in rice [43]. Many of these genes are expressed in both the roots and the shoots of the plants, namely *OsYSL6*, *OsYSL14*, *OsYSL16* while others are expressed preferentially in the shoots such as *OsYSL13* and *OsYSL2*. Further characterization of OsYSL2 has revealed that OsYSL2 is a plasma membrane protein, and in *Xenopus*, OsYSL2 is capable of mediating the transport of Fe(II)–NA, Mn(II)–NA but not of Fe(III)–DMA or Mn(II)–DMA. *OsYSL2* mRNA accumulates to higher levels in Fe-deficient roots than in Fe-sufficient roots. At the tissue level, *OsYSL2* is expressed in the companion cells of the phloem suggesting a role for OsYSL2 is the transport of Fe and Mn in the phloem.

NA, a non-protein amino acid found in all plants and a precursor to MA, is thought to function in the intercellular transporter of Fe in both Strategy I and Strategy II plants. Supporting this, the *chloronerva* (*chln*) mutant from tomato, which is unable to synthesize NA, also shows intercostal chlorosis similar to the *ys-1* mutant phenotype [44,45]. In addition, NA appears to be required for intracellular transport as well. It seems that NA is needed to form a complex with Fe in order to keep it in a soluble form and that NA is needed for the intracellular transport of Fe to proteins responsible for regulating Fe uptake genes. Specifically, NA is required for the down-regulation of *Leirt1* and *Lenramp1* transcription under Fe-sufficient conditions [46]. More specifically, although *Leirt1* and *Lenramp1* are induced by low-Fe conditions in wild type and the mRNA levels decrease with Fe supply, in the *chln* mutant, the mRNAs of these genes accumulate to lower levels in low Fe and their accumulation increases with additional supply of Fe. One possibility is that this result is due to the induction of *IRT1* and *FRO2* by local iron (discussed below). More specifically, the *chln* mutant could be more Fe-deficient than wild type. Thus, when Fe is added, *chln* may remain Fe-deficient and Leirt1 and Lenramp1 may be induced by the addition of Fe. In contrast, the addition of Fe to wild type may alleviate the Fe deficiency thereby downregulating the Fe deficiency responses. When *chln* was cloned [47], it was shown to encode the enzyme nicotianamine synthase (NAS) based on sequence similarity to NAS proteins from barley [47,48]. *chln* is expressed in both roots and shoots regardless of Fe status [47].

2.4. The regulation of Fe deficiency responses

Much of what is understood about the regulation of Fe deficiency responses in Strategy I plants comes from a tomato mutant, *fer*, characterized by its inability to induce Fe deficiency responses [49,50]. Fe uptake in this mutant is thus blocked, and it is a lethal phenotype. *FER* was recently cloned and its mRNA is detected specifically in the epidermal cells of roots as well as the outer cortical layer of root tips [51]. In addition, *FER* mRNA can be seen in the vascular cylinder of the mature root-hair zone. Although the accumulation of *FER* mRNA does not appear to be regulated by Fe availability, FER protein levels are controlled by Fe availability [52]. When *FER* is expressed under the control of the strong, constitutive CaMV 35S promoter, *FER* mRNA can be detected in roots regardless of Fe status. FER protein, however, is down-regulated at elevated Fe levels.

FER encodes a bHLH transcription factor suggesting that it may act as an Fe sensor to control Fe deficiency responses in the root. Accordingly, the mRNA expression profiles of *Lenramp1* and *Leirt1* are both altered in the *fer* mutant [46]. More specifically, while in wild type both of these genes are root-specific and induced by low Fe conditions, in the *fer* mutant, the mRNAs of these genes are absent regardless of the Fe status of the plant. However, the ectopic expression of *FER* is insufficient to drive the expression of either gene indicating that FER is necessary but not sufficient for *Lenramp1* and *Leirt1* expression.

A protein with high similarity to FER has also been characterized in Arabidopsis [8,53,54]; expression of FIT1 (also known as *bHLH29/FRU*) in the *fer* mutant of tomato rescues the ability of these plants to induce the Fe deficiency responses [54]. Like *FER*, *FIT1* is also root-specific, however, *FIT1* is induced under Fe-deficient conditions. *FIT1* has also been localized to the epidermal cells of Fe-deficient plants but, unlike *FER*, has not been seen in the vasculature. *fit1* mutants, like *fer* mutants, are chlorotic and die before setting seed unless supplied with Fe, a phenotype similar to that of *irt1* plants [18–20]. This is perhaps not surprising because IRT1 protein is absent in *fit1* mutants although *IRT1* mRNA is still present [8]. The mRNA of *FRO2* is absent in *fit1* mutants as is Fe(III) chelate reductase activity [8]. At this point, although it is clear that FIT1 alone does not directly control the transcription of *IRT1*, it is possible that *FRO2* is a direct target of FIT1. However, in plants overexpressing *FIT1*, both *FRO2* and *IRT1* mRNA levels are basically unchanged compared to wild type, suggesting that even if *FRO2* is a direct target of FIT1, FIT1 alone is not sufficient to regulate *FRO2*. One possibility is that FIT1 needs to heterodimerize with another factor in order to regulate *FRO2*.

IRT1, like *FRO2*, is also regulated post-transcriptionally [55]. The mechanism of this regulation is currently not known, but it is presumed to be analogous to the regulation seen for Zrt1p (*Z*inc-*r*egulated *t*ransporter) which, in the presence of Zn,

is ubiquitinated on a lysine residue within the variable region, K195 [56–58]. This ubiquitination triggers the subsequent endocytosis and degradation of Zrt1p in the vacuole [56]. Although *IRT1* mRNA can be detected in the roots and shoots of *IRT1* overexpressing plants regardless of Fe status, IRT1 protein can only be detected in Fe-deficient roots; this poses a problem for the use of IRT1 in increasing Fe levels in plants. Additionally, IRT1 can mediate the transport of the toxic metal Cd *in planta* thus requiring that the specificity of the transporter be enhanced prior to it being an appropriate target for biotechnology [18,55]. Fortunately, the specificity of IRT1 has been shown to be enhanced by single amino acid substitutions [59].

One complicating feature of *IRT1* and *FRO2* expression is their induction by local Fe [60]. In order to demonstrate this, the authors performed an experiment in which they grew plants under Fe-deficient conditions before transferring the plants into a split root system. Half of the root system was kept Fe deficient while the other half was supplied with Fe. In this system, *IRT1* and *FRO2* mRNAs were shown to be more abundant in the presence of Fe. Additionally, Fe(III) chelate reductase activity was also higher. Presumably, the initial growth under Fe-deficient conditions induced a shoot to root signal that caused the mRNA accumulation of *IRT1* and *FRO2* in the roots. In the presence of this signal together with local Fe, *IRT1* and *FRO2* mRNA accumulated to higher levels than in the presence of the signal without local Fe. Theoretically, it would be beneficial to the plant to only express *IRT1* and *FRO2* if Fe were in fact present to be mobilized. However, it is not clear how these split-root experiments fit with the post-transcriptional regulation seen for both IRT1 and FRO2. One possibility is that the levels of Fe supplied in the split-root experiments simply were not high enough to induce the post-transcriptional regulation of these genes.

2.5. Perceiving and relaying the Fe status of the plant

Some questions in Fe homeostasis that still need to be answered are how the plant is able to perceive and signal information regarding the Fe status of the plant as well as how Fe is distributed throughout the plant. One mutant that has helped elucidate the mechanisms of Fe localization within the plant is the *frd3* mutant of Arabidopsis. *frd3* mutants have deregulated Strategy I responses and thus accumulate more Fe despite their chlorotic appearance [61]. However, the increased Fe content of *frd3* plants is due to the accumulation of apoplastic Fe because *frd3* protoplasts contain approximately half as much Fe as wild-type protoplasts [62]. Therefore, one possibility is that *frd3* mutant plants constitutively express Strategy I responses because the shoots of *frd3* plants are functionally Fe deficient and not because there is a root defect in regulating Strategy I responses. In other words, the phenotype of *frd3* plants may arise in part due to a defect in the uptake of Fe within the shoots. However, when *frd3* shoots are grafted onto wild-type roots, the *frd3* shoots are capable of regreening. This result together with other grafting combinations demonstrates that the *frd3* phenotype is controlled by the genotype of the roots. Consistent with this, *FRD3* is expressed specifically in roots under both Fe-deficient and Fe-sufficient conditions and belongs to the *MATE* (*M*ultidrug *A*nd *T*oxin *E*fflux) family. Although this family is largely uncharacterized, several family members play a role in the efflux of small molecules from the cytoplasm to the outside of the cell or into the vacuole (as cited in [61]). *FRD3* mRNA localizes to the pericycle and vascular cylinder, and FRD3 protein localization using a GFP fusion protein is consistent with at least some FRD3 protein localizing to the plasma membrane. If FRD3 functions in export, it could function to efflux either Fe or a Fe chelator into the vascular cylinder. It does not appear that FRD3 transports Fe, however, because *frd3* mutants accumulate high levels of Fe(III) in the xylem of the roots compared to wild type. Therefore, the most likely role for FRD3 is in the export of a Fe chelator which is necessary to provide the shoots with a usable form of Fe.

3. Zinc

Zinc is an essential co-factor required for the structure and function of numerous proteins. In plants, Zn deficiency is one of the most widespread mineral deficiencies and may be the most common mineral deficiency in cereals [6,63]. As described above, plants use different strategies to obtain Fe from the soil. Unlike Fe, Zn does not need to be reduced before transport. The grasses, which extrude PS in response to Fe deficiency, may also use this chelation strategy in order to obtain Zn from the soil [64]. Consistent with a role in Zn uptake in the grasses, Zn–PS complexes have been demonstrated to be taken up by maize [65]. In addition, several Zn-efficient cultivars exist. Zn-efficient plants are more tolerant to Zn deficiency than non-efficient cultivars, and one proposed explanation for this is that Zn-efficient cultivars have an increased ability to obtain the limiting nutrient rather than an increased tolerance to lower levels of the nutrient [63,66]. Although the molecular mechanisms for Zn efficiency are not understood, it has been suggested that it arises from an increased secretion of PSs by Zn-efficient plants. Several reports demonstrate an increase in PS extrusion under Zn-deficient conditions [67–69], although it has also been suggested that the reproducibility of this extrusion is largely dependent on the growth conditions [70].

There are currently conflicting results on whether YS1 can mediate the transport of Zn–PS complexes [71,72]. However, YS1 protein accumulates under Fe-deficient conditions but is not detectable under Zn-deficient conditions, suggesting that YS1 functions mainly in Fe transport [72]. Furthermore, several ZIP proteins (described below) have been characterized in the Strategy II plant, rice, suggesting that this family plays a role in Zn acquisition in the grasses [73].

3.1. The role of the ZIP family of metal transporters in Zn transport

There are over 100 members of the ZIP family found at every phylogenetic level including archaea, bacteria, fungi, plants and mammals. 16 of these members are from Arabidopsis [17,74]. The Arabidopsis ZIP1, ZIP2, ZIP3 and ZIP4 proteins can all

functionally complement a yeast strain defective in Zn uptake ([75] and Guerinot lab, unpublished data). In planta, the mRNAs of *ZIP1*, *ZIP3*, and *ZIP4* accumulate in response to Zn deficiency. *ZIP1* and *ZIP3* are root specific while *ZIP4* mRNA accumulates in both the roots and shoots of Zn-deficient plants. *ZIP4* mRNA also appears to be Cu regulated [76]. Furthermore, ZIP4 is able to complement the Cu uptake defect of a *ctr1* (*c*opper *tr*ansporter) mutant in yeast suggesting that ZIP4 can in fact transport Cu.

The soybean homolog of ZIP1, GmZIP1 appears to function in the symbiosis between soybean and *Bradyrhizobium japonicum* [77]. In addition to complementing a Zn uptake mutant of yeast, the protein localizes to the peribacteroid membrane, and *GmZIP1* mRNA is seen specifically in nodules. Furthermore, antibodies to GmZIP1 inhibited Zn uptake into symbiosomes. Interestingly, because uptake into the symbiosome is equivalent to transport out of the cell, it appears that GmZIP1 transports in a direction different than that of other characterized ZIP family members.

Several ZIP proteins have been characterized in rice [23,73,78]. *OsZIP4* mRNA accumulates in Zn-deficient shoots and roots [78]. The expression of *OsZIP4* in yeast defective in plasma membrane Zn uptake restores the ability of these yeast to grow under Zn-limited conditions. OsZIP4 presumably functions to transport Zn across the plasma membrane into the cytoplasm based on the fact that when OsZIP4 is fused to GFP and transiently expressed in onion epidermal cells, fluorescence is detected at the plasma membrane. In rice OsZIP4 may be involved in the translocation of Zn throughout the plant; in situ hybridization experiments revealed that *OsZIP4* mRNA accumulates in the phloem cells of the stem as well as in the vascular bundles of the roots and leaves. *OsZIP1* and *OsZIP2*, which are most closely related to Arabidopsis ZIP2, are more highly expressed in response to Zn deficiency [73]. *OsZIP1* mRNA accumulates in Zn-deficient roots and shoots while *OsZIP2* mRNA accumulates primarily in Zn-deficient roots. A protein similar to ZIP2 has also been characterized in *Medicago truncatula* [79]. MtZIP2 is able to complement the Zn-uptake mutant of yeast, and the protein localizes to the plasma membrane of onion epidermal cells. *MtZIP2* mRNA, however, is upregulated by Zn fertilization. Likewise, colonization of roots with arbuscular mycorrhizae, which can reduce plant Zn levels, causes the down-regulation of *MtZIP2*.

3.2. The role of the CDF (Cation Diffusion Facilitator) family of metal transporters in Zn transport

Members of the CDF family are found in bacteria, fungi, plants and animals [80] and function in transporting metals from the cytoplasm, either by effluxing it to the extracellular medium or by transporting it into intracellular organelles or storage compartments [81,82]. Although there are twelve predicted CDF family members in Arabidopsis [17], only one, MTP1 [(*M*etal *T*ransport *P*rotein) which is also known as *ZAT1* (Zinc transporter of *Arabidopsis thaliana*)], has been characterized to date [83–85]. *MTP1* mRNA is present in all tissues and the addition of Zn does not induce higher levels of expression. When *MTP1* is overexpressed, however, plants become resistant to toxic levels of Zn. This resistance appears to be due to an increased sequestration of Zn rather than an efflux mechanism because the roots of overexpressing plants actually contain more Zn than wild-type plants. Consistent with this, MTP1 has been localized to the vacuolar membrane [84,85]. The phenotype of *MTP1* knock down or knock out plants also supports a function for MTP1 in the transport of Zn into the vacuole as they are highly sensitive to increased Zn levels [84,85]. Heterologously expressed MTP1 protein can bind Zn in a metal blot and can mediate the transport of Zn, but not Co, into proteoliposomes [86]. Furthermore, the transport of Zn into proteoliposomes does not require a proton gradient, suggesting that MTP1 either does not use an antiport mechanism for transport or that it uses another cation such as K. Although MTP1 could not complement the *zrc1* (*z*inc *r*esistance *c*onferring) mutant in *S. cerevisiae*, it was able to complement the Zn-sensitive phenotype of the *Schizosaccharomyces pombe zrc1* mutant [84,86]. In addition, MTP1 can complement the *zrc1cot1* (*co*balt *t*ransporter) double mutant of *S. cerevisiae* as well as mediate the efflux of Zn in *Xenopus* oocytes [84,87]. The expression of *MTP1* in the bacterium *Ralstonia metallidurans* also demonstrated that MTP1 can function in Zn efflux [86].

While no other Arabidopsis CDF proteins have been characterized, several CDF genes from other plants have been heterologously expressed and characterized in Arabidopsis. For example, overexpression of the poplar *PtdMTP1* gene in Arabidopsis conferred Zn tolerance [88]. Additionally, the expression of *PtdMTP1* in yeast could rescue the Zn-sensitive phenotypes of *zrc1* or *cot1* but could not reverse Co, Cd, Mn or Ni sensitivity. Using a GFP fusion, the authors demonstrated vacuolar localization suggesting that PtdMTP1 functions by transporting Zn from the cytoplasm into the vacuole. Furthermore, the expression of a CDF family member from *Stylosanthes hamata*, ShMTP1, in yeast and Arabidopsis caused Mn to be sequestered into organelles thereby conferring resistance to elevated Mn [89]. *Stylosanthes hamata* is a tropical legume that is able to grow in Mn-rich soils [90], and the resistance to Mn is specific; the expression of ShMTP1 in yeast does not confer resistance to Al, Cd, Ca, Co, Cu, Hg, Ni or Zn [89].

3.3. The role of the HMA (Heavy Metal ATPase) family of metal transporters in Zn transport

Two members of the HMA family, HMA2 and HMA4, have recently been demonstrated to function in Zn homeostasis in Arabidopsis [91]. HMA2 and HMA4 belong to an eight member family of type P_{1B}-type ATPases in Arabidopsis [91,92]. The expression of *HMA4* in a Zn-sensitive *E. coli* mutant restores Zn tolerance [93]. In addition, while the *hma2*, *hma3*, *hma4* single mutants and the *hma3 hma4* double mutants do not have any obvious growth defects when grown in soil, *hma2 hma4* double mutants are chlorotic and fail to set seed [94]. Interestingly, this growth defect can be suppressed by the addition of excess Zn to the growth medium despite the fact that these double mutants consistently have lower levels of Zn in the

aerial portions of the plant even when excess Zn is added. This result suggests that there is minimum threshold of Zn required for growth. Despite the decrease in Zn in aerial portions of the plant, the roots of these plants have approximately 2-fold more Zn than wild type. Therefore, it appears that these mutants have a defect in translocating Zn from the roots to the shoots of the plant. Consistent with this, promoter GUS fusions show that these genes localize to the vascular bundles in the roots and the shoots of the plant, and HMA2 and HMA4 have both been demonstrated to be plasma membrane proteins [94,95]. Finally, both of these proteins appear to function in Cd transport within the plant as well because mutations in either enhance the Cd-sensitive phenotype of *cad1* mutants [94]. The enhanced susceptibility to Cd suggests that HMA2 and HMA4 may function to transport Cd to a specific tissue or organelle for sequestration and/or detoxification.

Despite the fact that neither the *hma2* nor the *hma4* single mutant displayed any obvious growth defects in soil, further investigation has revealed that both single mutants have defects in Zn and Cd homeostasis. Elemental analysis of whole plants shows that *hma2* mutants accumulate more Zn and Cd than wild-type plants, although they do not appear to have an increased sensitivity to either metal [96]. *hma4* mutant plants accumulate more Zn and Cd in the roots but they accumulate less Zn and Cd in leaves [95]. Consistent with this observation, the *hma4* plants have an increased sensitivity to both Cd and Zn [97]. Conversely, plants overexpressing *HMA4* have an increased tolerance to both Zn and Cd [95]. Several lines of evidence support a role for HMA4 in mediating the efflux of Zn across the plasma membrane. First, In addition to localization to the PM, expression of *HMA4* in wild-type yeast results in a decreased accumulation of radiolabeled Cd and radiolabeled Zn [97].

3.4. The expression of Zn transporters in hyperaccumulating plants

Perhaps not surprisingly, members of all of the gene families previously discussed in this section have been found to be more highly expressed in hyperaccumulators. Hyperaccumulators are plants that are able to accumulate levels of metals that are toxic to their non-hyperaccumulating counterparts. More specifically, *Thlaspi caerulescens* and *Arabidopsis halleri* are both Zn and Cd hyperaccumulators [98,99]. ZNT1 from *T. caerulescens* is most closely related to ZIP4 from *A. thaliana* [100]. *ZNT1* mRNA is abundant in *T. caerulescens* regardless of Zn status suggesting that ZNT1 plays a role in the ability of *T. caerulescens* to accumulate metals [101]. In contrast, in the non-hyperaccumulating species, *T. arvense*, *ZNT1* mRNA accumulates only under Zn-deficient conditions. ZNT1 can mediate the transport of both Zn and Cd in yeast [100].

In *Arabidopsis halleri*, *ZIP6* and *ZIP9* appear to be more highly expressed than in *A. thaliana*. Two groups interested in genes involved in the ability of hyperaccumulators to take up and/or detoxify metals used microarray profiling to look for genes that were more highly expressed in the roots or shoots of *A. halleri* as compared to *A. thaliana*. The experiments were performed using *A. thaliana* gene chips that represent approximately one third of the genome. The study focused on shoots identified *ZIP6* as being more highly expressed in *A. halleri* [102]. Real time RT-PCR experiments demonstrated that *ZIP6* was expressed 23- to 24-fold higher in the shoots of *A. halleri* than *A. thaliana*. The expression in roots was also increased, being 4- to 9-fold higher. This same study also identified *AhHMA3* as being highly expressed in the shoots of *A. halleri*. In addition, the authors demonstrated that the expression of *AhHMA3* in a *zrc1 cot1* double mutant of yeast could improve the Zn tolerance of this mutant suggesting that AhHMA3 may play a role in Zn detoxification. In the roots of *A. halleri*, ZIP9 was identified as being expressed 43-fold higher relative to *A. thaliana* [103]. Using RT-PCR the authors demonstrated that *ZIP9* increases in response to low Zn in both *A. halleri* and *A. thaliana*.

A member of the CDF family, *AhCDF1–3* (also known as AhMTP1), which is similar to MTP1/ZAT1 of *A. thaliana*, is expressed approximately 10- to 20-fold higher in the shoots of *A. halleri* compared to *A. thaliana* [102]. In addition, when expressed in yeast, *AhCDF1–3* is able to suppress the Zn-sensitive phenotype of the *zrc1 cot1* double mutant. AhCDF1–3 was also identified in a screen for cDNAs which, when expressed in a *zrc1 cot1* mutant, could restore resistance to Zn [104]. AhCDF1–3 localizes to the vacuolar membrane in yeast as well as in Arabidopsis protoplasts. Although the activity of this protein may also be higher in *A. halleri* than in the non-hyperaccumulating *A. thaliana*, it appears that it is increased at least in part at the level of expression. More specifically, while it appears to be present only as a single copy in *Arabidopsis thaliana*, it is present in at least four copies in *A. halleri*. While the expression of the CDF gene *ZTP1* (ZAT gene from *Thlaspi caerulescens*) is observed in several *T. caerulescens* accessions, it is highest in the accession that has the highest tolerance to Zn [105].

In *Thlaspi goesingense*, which hyperaccumulates Ni, TgMTP1 was identified in a search for putative vacuolar ion transport proteins [106,107]. In more recent work, several allelic variants of TgMTP1 were identified [108]. The authors amplified the *MTP1* gene from a single plant, cloned and sequenced different variants, of which three are considered to be true alleles of *MTP1* (TgMTP1a–TgMTP1c). Interestingly, each of these three alleles conferred similar resistance to Zn when expressed in the *zrc1 cot1* mutant of *S. cerevisiae*. In order to define the functional domains of the protein, the authors expressed truncated versions of TgMTP1b in the *zrc1 cot1* mutant and found that all portions of the protein are required for Zn resistance. Although a TgMTP1∷HA fusion protein localized to both vacuolar and plasma membranes in yeast, TgMTP1 is believed to function on the plasma membrane. This is based on the fact that the Zn resistance observed in yeast expressing TgMTP1 appears to be happening independently of the vacuole and that *zrc1 cot1* yeast expressing TgMTP1 show reduced Zn levels suggesting that TgMTP1 functions to efflux Zn from the cells rather than to sequester Zn in the vacuole. Further support of a function for TgMTP1 in efflux

across the plasma membrane comes from the localization of a TgMTP1::GFP fusion to the plasma membrane in Arabidopsis protoplasts.

In addition to the metal transport families discussed above, another type of metal transporter has been characterized in *A. halleri* [109]. *AhMHX1* was cloned based on homology to *AtMHX1*, a vacuolar transporter capable of exchanging protons with Mg, Zn and Fe in order to sequester these metals in the vacuole. Using antibodies directed against AtMHX1, AhMXH1 is detected in the leaves of *A. halleri* plants. Interestingly, although the transcript levels of *MHX1* are similar in *A. thaliana* and *A. halleri*, the protein accumulates to higher levels in *A. halleri* than in *A. thaliana*. This suggests that there is post-transcriptional regulation, but the mechanism of this regulation has not yet been elucidated. Finally, a mutant of *Medicago truncatula* has been identified, *raz* (*r*equires *a*dditional *Z*n), that appears to affect the subcellular distribution of zinc but the molecular nature of the mutation is unknown [110].

4. Copper

Cu is an essential micronutrient for plants due to the roles it plays as a cofactor in enzymes involved in both respiration and photosynthesis, yet, like other redox-active metals, Cu is toxic at elevated concentrations. Therefore, plants and other organisms regulate their intracellular Cu levels by regulating the uptake of Cu as well as well as by minimizing free Cu concentrations within the cell through metallochaperones [111,112]. Metallochaperones are soluble Cu binding proteins that serve to bind Cu and deliver it to sites where it is needed within the cell.

4.1. The role of the HMA family in Cu transport

Two types of Cu transporters have been identified thus far in plants. The first class is P-type ATPases belonging to the HMA family. RAN1 (*R*esponsive-to-*AN*tagonist) (also known as HMA7), a P-type ATPase similar to human Menkes/Wilson proteins and Ccc2p from *S. cerevisiae*, was isolated in a genetic screen for plants that exhibited constitutive ethylene responses [113]. The expression of RAN1 or the RAN1 ortholog from *Brassica napus*, BnRAN1, can functionally complement the *ccc2* mutant of *S. cerevisiae* [113,114]. Ccc2p acts to transport Cu into the secretory pathway thereby providing a necessary cofactor for the Fet3p oxidase, and *ccc2* mutants, therefore, lack oxidase activity. However, when alleles harboring the same missense mutations found in *ran1* mutants were expressed in *ccc2*, oxidase levels were not restored, implying that the mutations impaired the Cu transport activity of the RAN1 protein [113]. In addition to transporting Cu in yeast, RAN1 most likely functions to transport Cu *in planta* as well. The mutant phenotype of *ran1* mutants can be rescued by the addition of Cu to the growth medium. Because the ethylene receptor, ETR1 (*Et*hylene *R*esponse), requires Cu as a cofactor, it is thought that the *ran1* phenotype is due to a failure to deliver Cu to the ETR1 receptor [113,115]. Based on analogy to Ccc2p, which localizes to the membranes of post-Golgi vesicles, RAN1 is thought to localize to the Golgi and load ETR1 with Cu. However, ETR1 localizes to the endoplasmic reticulum [116]. Therefore, the localization of RAN1 and where it functions *in planta* remains an open question.

A second P-type ATPase has been characterized in Arabidopsis, PAA1 (*P*-type *A*TPase of *A*rabidopsis)(also known as HMA6) [117]. In a screen for high chlorophyll fluorescence phenotypes, six alleles of *paa1* were identified. These mutants have a defect in photosynthetic electron transport that can be suppressed by the addition of Cu. PAA1 has a functional chloroplast targeting signal, and *paa1* mutants have decreased chloroplast Cu content despite having normal Cu levels in the shoots, suggesting a defect in the delivery of Cu to the chloroplasts. Consistent with this, *paa1* mutants lack functional plastocyanin which requires Cu as a cofactor. While holoplastocyanin (containing Cu) was only detected at very low levels in *paa1* mutants, apoplastocyanin (lacking Cu) accumulated to higher levels than in wild type. In addition, the activity of a Cu/Zn superoxide dismutase, which localizes to the chloroplast and requires Cu, is also decreased in *paa1* mutants. Together these data suggest that PAA1 functions to transport Cu into the stroma of the chloroplast.

In a similar screen for high chlorophyll fluorescence phenotypes, *paa2* was identified [118]. The *PAA2* (also known as *HMA8*) gene encodes a metal-transporting ATPase similar to PAA1. Like *paa1* mutants, the phenotype of *paa2* mutants can be suppressed by the addition of Cu. Similarly, the limitation of Cu enhances the high fluorescence phenotype. Although neither *paa1* nor *paa2* mutants display a lethal phenotype, *paa1 paa2* double mutants die when grown in soil. In order to further understand the roles of PAA1 and PAA2, the authors constructed PAA1::GFP and PAA2::GFP fusions. In protoplasts expressing *PAA1::GFP*, fluorescence was detected at the perimeter of the chloroplasts suggesting that PAA1 functions to transport Cu across the envelope of the chloroplast. Although the authors were unable to localize full length PAA2 fused to GFP, when the predicted transit signal peptide of PAA2 is fused to GFP, fluorescence is detected in the chloroplasts. Based on the activity of several enzymes that require Cu for their activity, PAA2 appears to be active on the thylakoid membrane. More specifically, plastocyanin is found in the thylakoid lumen while CSD2, which also requires Cu for activity, is active in the stroma. Both *paa1* and *paa2* mutants have reduced holoplastocyanin levels but CSD2 activity is decreased in *paa1* mutants and increased in *paa2* mutants. These data suggest that PAA1 functions to transport Cu across the chloroplast envelope into the chloroplast stroma while PAA2 functions to transport Cu across the thylakoid membrane, thereby transporting Cu out of the stroma and into the thylakoid lumen.

Two other members of the HMA family, HMA1 and HMA5, have been implicated in Cu transport [119,120]. HMA1, like PAA1 and PAA2, appears to be involved in Cu homeostasis in the chloroplast [120]. *hma1* mutants accumulate decreased levels of Cu in the chloroplasts, have decreased SOD activity and are sensitive to high light. Consistent with a role in Cu homeostasis in the chloroplast, HMA1 localizes to the

chloroplast envelope. Using the first 119 amino acids of HMA1 fused to GFP, the authors localized HMA1 to the chloroplast. The localization was further verified using antibodies directed against HMA1 on subplastidial protein fractions; HMA1 was detected in the envelope membrane protein fraction. When full-length *HMA1* was expressed in yeast, no increased tolerance to Zn, Cu or Co was detected. However, when a truncated version of HMA1 that lacked the first 60 amino acids was expressed, the protein was toxic to the yeast cells. Mutation of a residue known to be important for catalytic turnover and transport in other family members abolished the observed toxicity, suggesting that the toxicity was indeed the result of increased metal transport.

HMA5 also appears to be involved in the transport of Cu as *hma5* mutants are sensitive to increased levels of Cu. Because *HMA5* transcripts are detected primarily in the roots and flowers, HMA5 is predicted to function in effluxing Cu from the cytoplasm, particularly when Cu is present in excess. However, it is currently not known whether HMA5 functions to efflux Cu out of the cell or into an organelle for sequestration. Interestingly, it appears as though HMA5 may also be able to obtain Cu from Cu chaperones. Using a directed yeast-two hybrid approach, HMA5 was shown to interact with two Cu chaperones, ATX1 (*A*n*t*i-o*x*idant) and CCH (*C*opper *Ch*aperone).

4.2. The role of the COPT family in Cu transport

A second family of metal transporters, the COPT (*Cop*per *T*ransporter) family, was identified through functional complementation of a yeast mutant defective in plasma membrane Cu uptake, *ctr1 ctr3* [121]. The COPT proteins belong to the CTR Cu transport family that has members in mammals and in yeast [122]. Members of this family contain three predicted transmembrane domains, and some members are capable of multimerizing to potentially form a pore within the membrane through which Cu can pass [123]. COPT1 was the first member identified [124], and subsequent members were identified through database searches [121]. The expression of COPT1 and COPT2 can restore growth to the yeast Cu uptake mutant whereas COPT3 and COPT5 only support limited growth. Interestingly, the authors were unable to obtain COPT4 transformants. All the complemented strains were able to mediate the uptake of radiolabeled Cu. *In planta*, the mRNA levels of *COPT1* and *COPT2* were decreased in the presence of Cu, although the levels of *COPT3*, *COPT4* and *COPT5* were unaffected. None of the genes showed any change in mRNA levels in response to Cu chelation. This is in contrast to members of the CTR family from other organisms which accumulate in response to Cu deficiency. For instance, the expression of *CTR1* and *CTR3* from *S. cerevisiae* is mediated through the transcription factor Mac1p which is active under Cu-deficient conditions [125]. However, the Cu-deficient conditions used in this experiment were to incubate Arabidopsis rosette leaves in a Cu chelator for 18 h [121]. Therefore, it remains possible that if the plants were grown under Cu-deficient conditions an increase in *COPT* mRNA would be observed.

The overexpression of antisense *COPT1* mRNA has implicated COPT1 in Cu transport *in planta* [126]. Although these antisense lines still have wild-type levels of *COPT1* sense mRNA, when the levels of *COPT2* and the Cu metallochaperone *CCC2* were investigated, elevated mRNA levels of both were observed. Because the mRNAs of both *COPT2* and *CCC2* are decreased in the presence of Cu, the authors reason that these genes are elevated in the antisense lines due to a decrease in Cu levels. Consistent with this, radiolabeled Cu uptake was reduced 40–60% in the antisense lines for both short- and long-term levels. Short-term levels were measured by incubating seedlings with radiolabeled Cu for 30 min whereas long-term levels were determined by elemental analysis of four-week-old plants. In addition to a decrease in Cu levels, the antisense plants showed an increase in root length that could be suppressed by the addition of Cu but not by the addition of Fe or Zn. The length of wild-type roots could be increased in a dose-dependent manner by the addition of a Cu chelator to the growth medium, although no additional growth of the antisense lines was observed. Lastly, the *COPT1* antisense lines have an increased percentage of pollen abnormalities when plants are grown under standard nutrient conditions. This phenotype could also be suppressed by the addition of Cu, suggesting that the defect is in a failure to delivery Cu to the developing pollen.

4.3. The role of metallochaperones in Cu homeostasis

Several metallochaperones have been identified in plants as well. Metallochaperones are known to bind metals and to deliver them to specific proteins within cells. The first to be identified in Arabidopsis was CCH [127]. At the amino acid level, the predicted protein shows homology to Atx1p, a Cu chaperone in yeast. The yeast *atx1* mutant is unable to growth on Fe-limited medium due to a failure in delivering Cu to the multi-Cu oxidase necessary for high-affinity Fe uptake, and the expression of *CCH* in an *atx1* mutant can suppress the high-affinity Fe uptake defect. *In planta*, *CCH* mRNA is broadly expressed in tissues including the root, stem, leaf, inflorescence and silique. In addition, *CCH* mRNA accumulates to higher levels after the start of leaf senescence [127,128], and *CCH* mRNA levels decrease in response to Cu treatment [127]. If CCH functions *in planta* in a manner similar to that of Atx1p, the role of CCH would be in the delivery of Cu from the cytoplasm to the RAN1 transporter at the post-Golgi membrane [115]. This has not been directly determined, however. Interestingly, CCH has been implicated in intercellular Cu delivery [129]. More specifically, the protein localizes to some phloem cells of green leaf cells in Arabidopsis but is detected more broadly in the phloem of senescing leaves as well in phloem exudate. Despite the down-regulation of *CCH* mRNA by the addition of Cu, CCH protein remains stable. Based on these results, the authors suggest that CCH plays a role in recycling Cu from senescing leaves.

CCS1 is orthologous to the yeast Ccs1p/Lys7p that delivers Cu to Sod1p, a Cu/Zn superoxide dismutase [130]; when *AtCCS1* is expressed without its predicted transit sequence in the yeast *lys7* mutant, it can restore superoxide dismutase

activity. *In planta*, the exact role of CCS1 is not known, but it appears to function in the chloroplasts based on the expression of a 35S-CCS1::GFP fusion. *CCS1* mRNA accumulates in response to elevated Cu conditions. Thus, one proposed role for CCS1 is in maintaining proper Cu levels for both plastocyanin and superoxide dismutases known to function in the chloroplast, such as CSD1 and CSD2.

Another Cu chaperone, COX17 (*C*ytochrome *Ox*idase), was identified using differential display as a gene induced after inoculating tobacco leaves with a protein elicitor from *Erwinia amylovora* [131]. The deduced protein shares homology to COX17 from yeast and a protein in Arabidopsis, AtCOX17. Defects in yeast *COX17* result in respiratory-deficiency due to a failure of the protein to deliver Cu to the mitochondrial cytochrome oxidase complex, and the expression of *AtCOX17* in this mutant background restores growth. *In planta*, the mRNA of *AtCOX17* is induced by pathogen infection and accumulates in response to the addition of various metals. The addition of Cu leads to the accumulation of *AtCOX17* with the highest expression levels observed in plants treated with the highest levels of Cu used in the experiment. In addition to Cu, Zn and Cd also induce the accumulation of *AtCOX17* mRNA, although at lower levels. Together these data suggest that AtCOX17 may function to deliver Cu to the mitochondria during times of stress that cause mitochondrial damage.

5. Concluding remarks

Although significant progress has been made in recent years in our understanding of how metals are obtained from the soil and distributed throughout the plant, there is still work to be done. Before we will be able to successfully engineer plants to improve human health, we need an integrated picture of all of the metal transport mechanisms. It is clear that within a multicellular organism such as a plant, the needs of each cell may be different. For example, the needs of a photosynthetically active cell are presumably different from those of a non-photosynthetically active cell. That said, all cells must be able to maintain proper metal homeostasis. So certain questions remain. Do all cells contain the same set of proteins involved in homeostasis or are these proteins differentially expressed to meet the demands of various cells? We know, for instance, that *NRAMP3*, *NRAMP4* and *VIT1* are all expressed in the vasculature of the roots and shoots, and the proteins that these genes encode appear to play a role in vacuolar Fe homeostasis. Presumably, vacuolar Fe homeostasis is a process required by all cells; are these genes really not expressed in other tissues or are they simply more abundantly expressed in the vasculature? Fig. 1 shows the subcellular localization of metal transporters based on GFP fusions and/or immunolocalization. Notice that only 14 of the more than 40 transporters discussed in this review are shown. It is clear that work remains to be done as the localization of every transporter needs to be known in order to fully understand metal uptake and distribution.

Most of this review is focused on transporters given the recent progress in this area. We know much less about how metals are safely stored and accessed within a cell. Cu appears to be the only metal that uses metallochaperones for delivery. Given that Fe, like Cu, is redox active, one might have thought

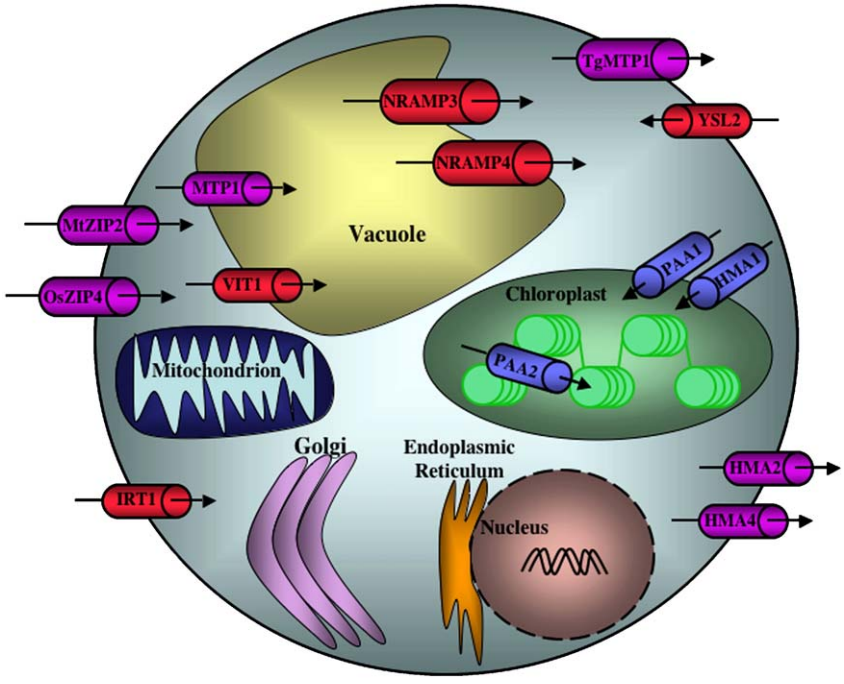

Fig. 1. Summary of the membrane distribution of Cu, Fe and Zn transporters localized in planta. Proposed Cu transporters are represented in blue, Fe transporters are shown in red and Zn transporters are shown in purple. All of the metal transporters shown have been localized in planta either using GFP fusions or immunolocalization. Arrows indicate the suggested direction of transport based on mutant phenotypes and expression in heterologous systems. For transporters known to transport multiple metals, only the proposed major metal substrate is shown.

that Fe would also need a chaperone. It is likely that most of the Fe in a cell is chelated but how proteins access this pool of Fe remains unknown. The intracellular and storage mechanisms for Zn are also areas requiring further study.

The community-wide effort to develop large T-DNA collections in recent years has greatly contributed to our understanding of how different transporters in the plant are functioning. Because we know that many of the transporters involved in uptake and distribution are members of gene families, combining loss-of-function mutations in different genes has played a critical role in elucidating the roles of HMA2 and HMA4 as well as NRAMP3 and NRAMP4. It will be exciting to see the individual roles of each transporter unravel in future years as more mutations are analyzed and combined. One thing we have learned from studies to date is that many of the transporters involved in Fe, Zn and Cu uptake can transport a variety of divalent cations but each transporter is usually transcriptionally and/or post-transcriptionally controlled by a particular metal.

One of the exciting prospects for the future will be in vivo imaging of individual metals *in planta*. Currently, the use of elemental analysis is fairly widespread in order to determine overall levels of metals in the plant; this has even been further reduced to look at the metal content of individual chloroplasts. What is needed, however, is to be able to look at individual cells. This has been done using electron microscopy coupled to imaging of inelastically scattered electrons (ESI) to look at the Fe mobilization from vacuolar globoids in plants [35]. In other organisms such as the budding yeast *Saccharomyces cerevisiae*, fluorescent metal binding dyes have been used to localize metal stores within cells. These techniques have not yet been extensively applied to plants, however. The ability to look at metal distribution at such a fine level would surely increase our ability to not only find phenotypes that are too difficult to see using current technologies but also to specifically understand the role each transporter is playing in cellular metal distribution.

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